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Introduction

Semaphorin 3B (*SEMA3B*) is located at 3p21.3, a site of very frequent allele loss in the early pathogenesis of lung and breast cancer (1-3). *SEMA3B*, encodes a secreted protein with tumor suppressor activity for lung cancer (4). Treatment with exogenously added *SEMA3B* or introduction of a plasmid encoding *SEMA3B* into H1299 non-small cell lung cancer (NSCLC) cells lead to induction of apoptosis and a dramatic decrease in colony formation (3, 4). Independent studies in ovarian cancer by Tse et al. (5) demonstrated that *SEMA3B* also inhibited ovarian tumor formation in a xenograft model. We previously hypothesize that inactivation of *SEMA3B* is a common and early even in breast and other cancers. Even further we hypothesize that *SEMA3B* exerts its tumor suppressor function through competing with breast cancer produced VEGF (vascular endothelial growth factor) acting as a tumor survival factor. If *SEMA3B* functions as a tumor suppressor that undergoes inactivation by mutation, LOH, promoter methylation or lost of receptors, it could provide both a diagnostic tool and as a soluble molecule a new treatment breast and other cancers with similar lost of *SEMA3B*.

Body

Research accomplishments associated with each task.

Task 1. Verification of tumor suppressor activity of *SEMA3B* in breast cancer cells.

Preparations of different expression vectors, p*SEMA3B*, Flag-*SEMA3B*, *SEMA3B* tumor acquired missense mutant and p53 have been accomplished. Proliferation studies in breast cancer cell lines are in process. We have observed 50-90% reduction in cell proliferation in around 75% breast cancer cell lines treated with Cos7 media after transfection with *SEMA3B*, or control vector (Figure 1). Major difficulties have been encounter with the liquid and soft agar colony formation assay for breast cancer lines. It is important to point out that the lung cancer line H1299 is used as a positive control for *SEMA3B* effect. The lung line H2009 which express endogenous levels of *SEMA3B* it was used as a negative control for *SEMA3B* effect. In conclusion we have found that most cells lines will respond to *SEMA3B* growth inhibition, but screening of a larger number of lines is necessary.

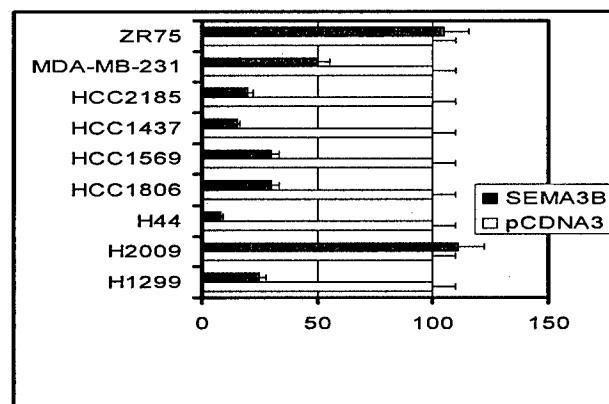
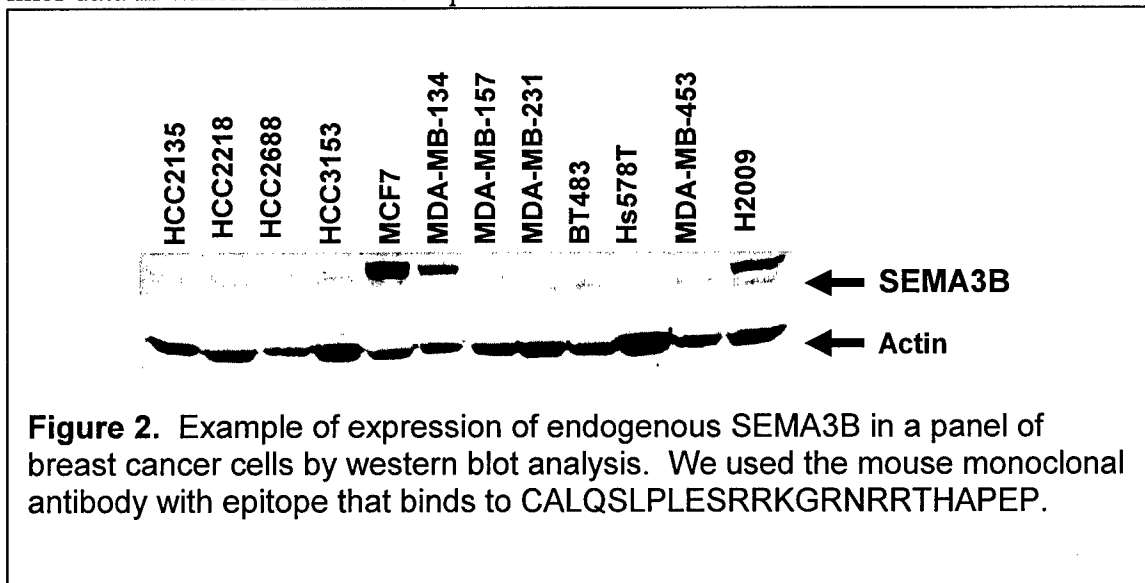


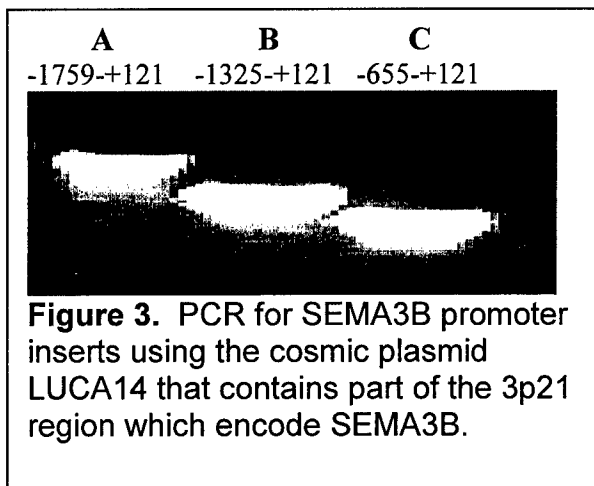
Figure 1. Example of effect of *SEMA3B* in cell proliferation in breast cancer cells lines. Cells were counted after 5 days of treated with *SEMA3B* or control media.

Task2. Study the expression and promoter methylation patterns of SEMA3B using a panel of breast cancer cell lines.

We are working in the development of a better method to distinguish promoter methylation in SEMA3B, the reason for this inconvenience is that SEMA3B promoter lacks of CpG islands, however contain C-rich regions. We have developed a SEMA3B antibody that can detect transfected protein and it is used as a blocking antibody against SEMA3B. We have also developed a monoclonal antibody that can detect endogenous levels of the SEMA3B protein. We used H2009 cells as a positive control for SEMA3B expression. We verified that the monoclonal antibody was targeting SEMA3B by using siRNA. After 72 hour post transfection of siRNA targeting SEMA3B we observed a more than 80% decreased in SEMA3B protein band intensity in H2009. In a panel of breast cancer lines for the presence of endogenous SEMA3B we found that only 20% express the endogenous protein (Figure 2). These data correlated with lung cancer cell lines data in which SEMA3B is expressed in less than 15% of the cell lines.



Task3. Study the SEMA3B promoter region and mechanism of its transcriptional activation.



We have made three SEMA3B promoter constructs in a pGL3 luciferase reporter vector. For the synthesis of SEMA3B promoter we used the cosmic plasmid LUCA14 (Figure 3) SEMA3B is a very C-rich promoter making its synthesis challenging.

The antiproliferative, apoptotic and cell differentiation effects of $1\alpha,25\text{-V}_3$ have been known for years. Studies in breast cancer cell MCF7 treated with the vitamin D analogue EB1089 demonstrated a correlation between

increased p21^{WAF-1/CIP1} protein levels and inhibition of Cdk2-associated histone H1 kinase

activity and G1 arrest (6). Further studies have shown EB1089 to decrease the level of c-myc mRNA and transiently increase c-fos with a 50 times more potency than $1\alpha, 25\text{-V}_3$ (7). Other studies link $1\alpha, 25\text{-V}_3$ -growth inhibition to TGF- β autocrine pathway (8).

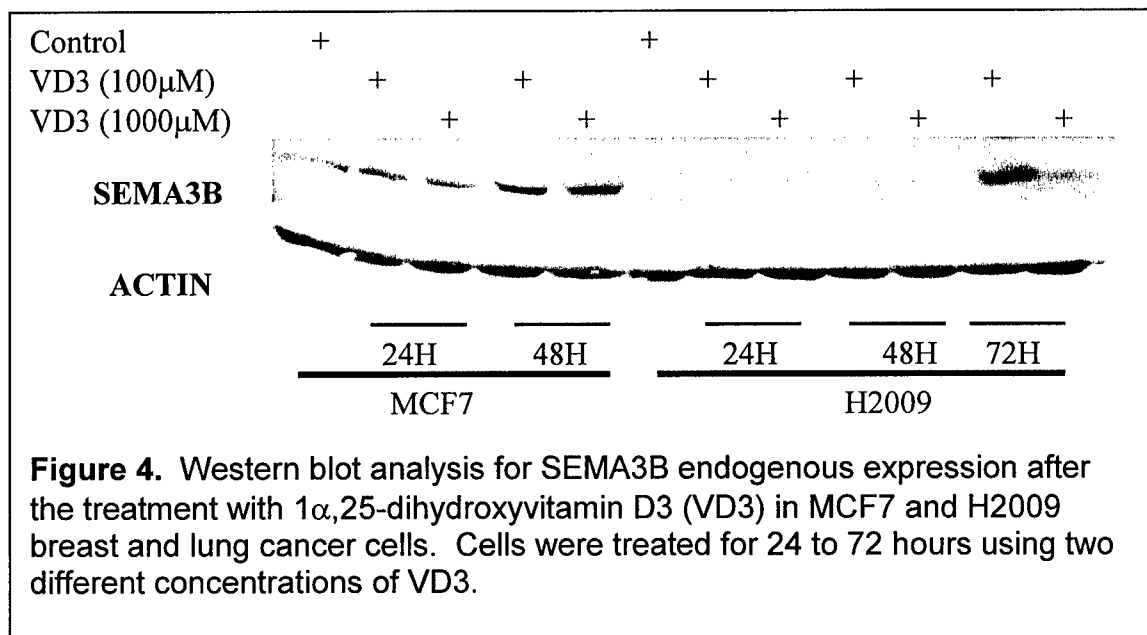


Figure 4. Western blot analysis for SEMA3B endogenous expression after the treatment with $1\alpha, 25$ -dihydroxyvitamin D3 (VD3) in MCF7 and H2009 breast and lung cancer cells. Cells were treated for 24 to 72 hours using two different concentrations of VD3.

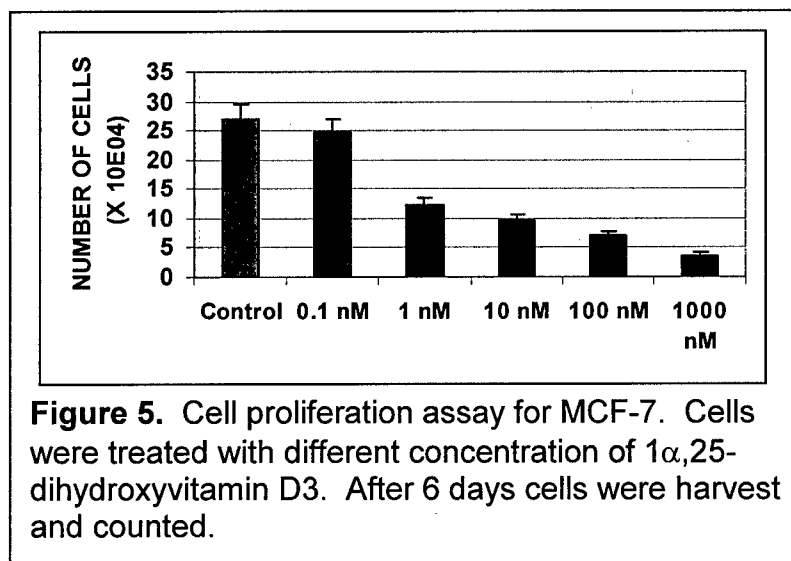
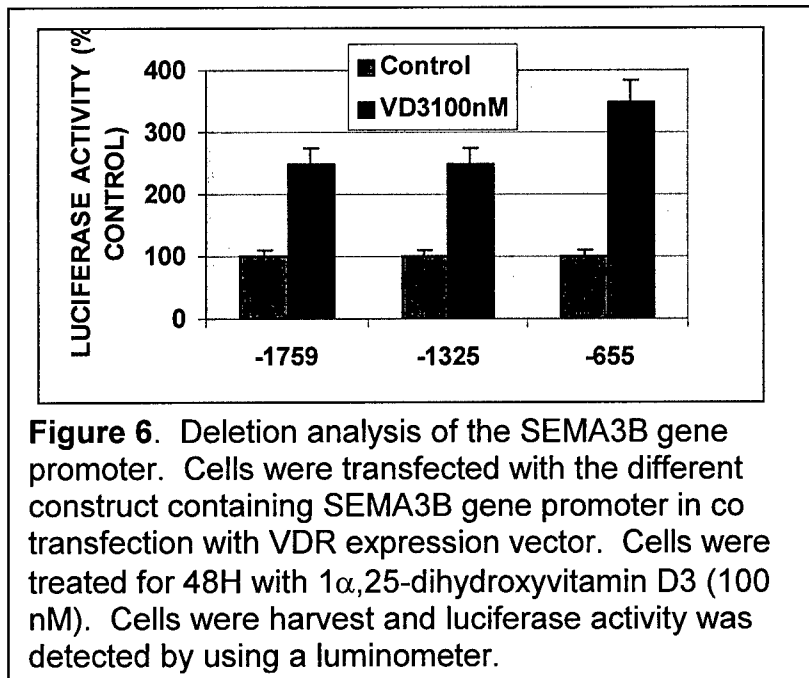


Figure 5. Cell proliferation assay for MCF-7. Cells were treated with different concentration of $1\alpha, 25$ -dihydroxyvitamin D3. After 6 days cells were harvest and counted.

Recently it was found that in squamous carcinoma cells showed that the active form of Vitamin D3, 1 α ,25-dihydroxyvitamin D3 and its receptor (VDR) is involve in SEAM3B transcriptional activation. MCF-7 treated with 1 α ,25-dihydroxyvitamin D3 showed an increased in SEMA3B protein after 48H (Figure 4), however in other experiments we has observed induction of SEMA3B protein as early as 6 hours (9). In western blot analysis we found that VDR is expressed in 65% of breast cancer lines (data not shown). MCF-7 showed endogenous expression of VDR and in proliferation studies we found a significant decreased in proliferation after treatment with 1 α ,25-dihydroxyvitamin D3 starting at concentration as low as 1 nM (Figure 5). In promoter studies for SEMA3B gene we used three different constructs -1759-+121, -1325-+121, -655-+121 links to a luciferase reported gene. We found that 1 α ,25-dihydroxyvitamin D3 at 100 nM induced the three construct by 2.5 to 3.5 fold when comparing with control or untreated cells (Figure 6). The next step it is to narrow down the sequence of 1 α ,25-dihydroxyvitamin D3 promoter activation and to determine if the activation it is by direct binding with the promoter or by activating other transcription factors.



Task 4. Study of the pathways and mechanism of tumor suppressor activity of SEMA3B in breast cancer cells.

Extensive work has gone to this task. Previous research has shown that heparin binding domain containing VEGF-A isoforms (145, 165, 189, but not 121) can bind to neuropilins (9). Neuropilins are the receptors for class 3 semaphorin (SEMA3B is part of this class). Further more resent research has shown that SEMA3A and VEGF₁₆₅ compete for binding with neuropilins. Firstly we determined the role of VEGF-A in the survival of H1299 lung and MDA-MB-231 breast cancer cell lines. We have used a anti-VEGF-A neutralizing antibody. Using these techniques that neutralize the biological activity of

VEGF-A, we observed a 40-80% decrease in cancer cell proliferation after four-five days of treatment (Figure. 7). These data suggest that tumor cell produced VEGF has an important role in the survival and or growth of H1299 lung and MDA-MB-231 breast cancer lines.

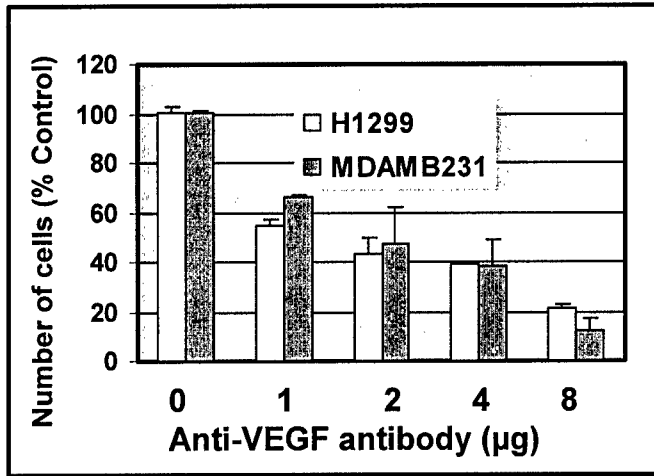


Figure 7. VEGF-A play a role in the survival of H1299 lung and MDA-MB-231 breast cancer cells. H1299 and MDA-MB-231 were seeded and treated with different doses of anti-VEGF monoclonal antibody. Cells were trypsinize and count after 4-5 days. The cell proliferation inhibition by the antibody was dose dependent.

Secondly we determined that SEMA3B inhibits ^{125}I -VEGF₁₆₅ binding to H1299 and MDA-MB-231 cancer cells. It is important to mention that our source of SEMA3B is from Cos7 cells medium after transfection with SEMA3B expression vector. Our control is the medium after Cos7 transfection with empty vector. Cell surface binding of ^{125}I -VEGF₁₆₅ was tested on both cell lines with and without the addition of SEMA3B. Specific ^{125}I -VEGF₁₆₅ binding was observed in both cell lines after two hour of incubation with 100 pM of labeled VEGF₁₆₅ (Fig. 8) SEMA3B, decreased the specific binding of ^{125}I -VEGF₁₆₅ of all cell lines (Fig. 8) with maximal inhibition of 50% for MDA-MB-231 and 65% for H1299, respectively. By contrast, Cos7 control had no significant effect on ^{125}I -VEGF₁₆₅ binding (Fig. 8). These data suggest that VEGF₁₆₅ and SEMA3B share binding sites on these human tumor cells as was previously observed for SEMA3A and VEGF₁₆₅ on NP receptor.

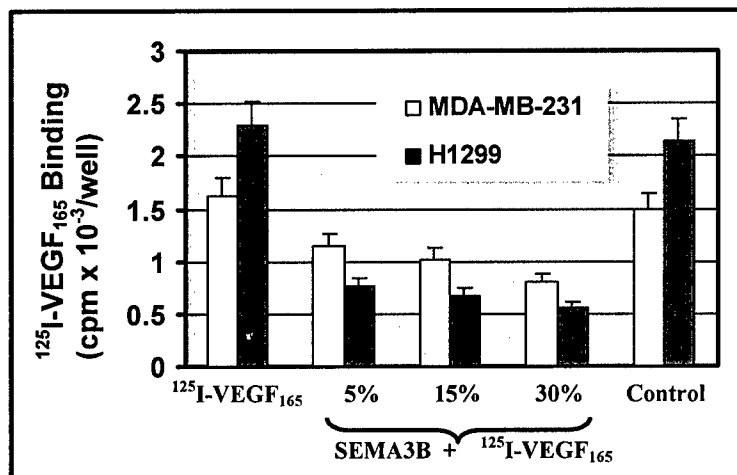


Figure 8. Competition of SEMA3B and ^{125}I -VEGF₁₆₅ for binding to the cell surface receptors on MDA-MB-231 and H1299 cells. MDA-MB-231 and H1299 were incubated for 2 hours with ^{125}I -VEGF₁₆₅ and SEMA3B in the binding buffer and the bound ^{125}I -VEGF₁₆₅ was detected using gamma counter. ^{125}I -VEGF₁₆₅ binding with SEMA3B added at 5, 15 and 30% of total volumen per well (200 μl) (lanes 2-4), which conditioned medium from the Cos-7 cells transfected with empty vector served as a control. Non-specific binding was determined in the presence of 100 fold molar excess of unlabeled VEGF unlabeled and was subtracted from the experimental values.

Thirdly, we determined that VEGF₁₆₅ antagonizes SEMA3B mediated effects on tumor cell proliferation. SEMA3B inhibited proliferation of H1299 and MDA-MB-231 cells by 50% compared with vector control while rabbit anti-SEMA3B antibodies neutralized this effect (Table 1). Control IgG from pre-immune rabbits had no effect on the inhibitory effect of the SEMA3B or vector control (data not shown). These results demonstrate that SEMA3B protein present in the control of SEMA3B transfected Cos7 cells, is responsible for the anti-proliferative effect. Treatment with mutant SEMA3B (SEMA3Bmut1) vector showed no effect in cell number when compared with control. The anti-proliferative effect of SEMA3B was overridden by co-treatment with of VEGF₁₆₅. By contrast, co-treatment with VEGF₁₂₁, which does not bind to NP-1 and NP-2, had no significant effect on cell proliferation as compared to SEMA3B alone. Transfection with VEGF₁₂₁ or VEGF₁₆₅ alone did not affect cell proliferation in the absence of SEMA3B. The specificity of the VEGF₁₆₅ reversal of SEMA3B growth inhibition was confirmed by treating with monoclonal anti-VEGF antibody 2C3 which detects VEGF bound to its receptors (10) which gave similar cell numbers as treatment with SEMA3B alone. In contract to the anti-VEGF neutralizing antibody the anti-VEGF monoclonal antibody 2C3 does not neutralize endogenous VEGF-A and no inhibition of growth was seen. The lacks of effect of VEGF₁₂₁ indicates that only a neuropilin-binding VEGF isoform (VEGF₁₆₅) is able to counteract the effects of SEMA3B. Taken together, these data suggest that SEMA3B is a potent growth inhibitory factor for human lung cancer H1299 and breast cancer MDA-MB-231 cells, and that VEGF₁₆₅ but not the VEGF₁₂₁ isoform, overcomes the SEMA3B growth inhibitory effect.

Table 1. Effect of SEMA3B-CM and VEGF isoforms 165 and 121 on proliferation in H1299 lung and MDA-MB-231 breast cancer cells.

Treatment ^a	H1299 Cell # (x10 ⁻⁴) (P value) ^b	MDA-MB-231 Cell # (x10 ⁻⁴) (P value) ^b
Control ^c	11 ± 1.32 (-)	16 ± 1.0 (-)
SEMA3B ^c	5.6 ± 0.57 (0.002) ^d	5 ± 2.0 (0.002) ^d
SEMA3Bmut ^e	11 ± 1.0 (0.0005) ^f	15 ± 0.5 (0.0005) ^f
SEMA3B + anti-SEMA3B ^g	10.8 ± 1.5 (0.004) ^f	13 ± 1.0 (0.004) ^f
VEGF ₁₂₁	10.7 ± 0.75 (NS) ^h	16 ± 1.0 (NS) ^h
VEGF ₁₆₅	11.15 ± 0.75 (NS) ^h	16 ± 2.0 (NS) ^h
VEGF ₁₂₁ + SEMA3B	6 ± 1.25 (0.004) ⁱ	8 ± 0.3 (0.003) ⁱ
VEGF ₁₆₅ + SEMA3B	12.3 ± 1.5 (0.0002) ^f	15 ± 0.6 (0.003) ^f
VEGF ₁₆₅ + anti-VEGF-A Ab	12 ± 0.9 (NS) ^j	14 ± 0.8 (NS) ^j
VEGF ₁₆₅ + SEMA3B + anti VEGF Ab ^g	6.6 ± 0.6 (0.001) ^k	4 ± 0.9 (0.001) ^k

^a Conditioned medium from cells transfected with various plasmids as specified above was added to MDA-MB-231 cells. ^b P value was calculated using two-tailed student t-test. ^c Cos7 cells were transfected with vector control or with SEMA3B expression vector and conditioned medium was collected 48 hours thereafter. ^d Significant decrease as compared to Control. ^e SEMA3B-mut (D397H) is inactive. ^f significant increase as compared to SEMA3B. ^g Anti-SEMA3B or anti-VEGF-A (30 µg/ml) antibody was used as neutralizing agents for SEMA3B and VEGF₁₆₅ respectively. ^h Non significant as compared to control. ⁱ Significant decrease when compared to cells treated with VEGF₁₂₁. ^j No significant as compared to VEGF₁₆₅. ^k significant decrease as compared to VEGF₁₆₅ + SEMA3B.

Key Research Accomplishments

- We made the expression plasmid for SEMA3B, SEMA3B-Flag (which it is partially inactive, SEMA3B with missense mutations (three different missense mutation found in lung cancer D397H, T415I, and D561N), and p53.
- Many breast and lung cancer lines showed a decrease in cell number after treatment with SEMA3B.
- SEMA3B mouse monoclonal antibody was made and was found to react with endogenous SEMA3B.
- SEMA3B expression is only found in 20% of breast cancer cell lines (30 lines were tested).
- SEMA3B gene promoter constructs were synthesis and introduce to a pGL3 luciferase reported gene vector.
- 1α,25-dihydroxyvitamin D3 induced SEMA3B in MCF7 breast cancer cells
- Vitamin D receptor is expressed in 65% of breast cancer cells
- 1α,25-dihydroxyvitamin D3 decreased cell number in proliferation studies in MCF-7 breast cancer cells
- 1α,25-dihydroxyvitamin D3 induces SEMA3B promoter up to 4 folds
- Neutralization of VEGF-A using anti-VEGF antibody produced a decrease in cell number showing the importance of VEGF-A in cell survival and growth for breast and lung cancer cells.
- SEMA3B could compete for VEGF₁₆₅ cell surface binding.

- VEGF₁₆₅ could negate the anti-proliferative effect of SEMA3B in breast and lung cancer cell but not VEGF₁₂₁.

Reportable outcomes

Manuscript

Castro-Rivera E., and Minna J.D., Induction of SEMA3B expression by vitamin D3 in MCF-7 breast cancer cells

Publication

Castro-Rivera, E, Ran, S, Thorpe, P and Minna, JD, Semaphorin 3B (SEMA3B) induces apoptosis in lung and breast cancer while VEGF₁₆₅ antagonizes this effect. PNAS 101(31) 2004.

Conclusion

Summary

In the present work we have shown that SEMA3B is expressed in more than 20% of breast cancer cell lines and inhibits cell proliferation in most of the breast cancer tested. Vitamin D3 induces endogenous SEMA3B and this induction was translated in SEMA3B gene promoter studies in MCF-7 cells. The significance of this work is that Vitamin D3 and vitamin D3 analogs are being studied for treatment of breast cancer, however vitamin D3 at higher concentration is toxic so maybe SEMA3B can become an alternative for vitamin D3 and its analogs treatment.

We also have shown that neutralizing anti-VEGF antibody inhibited breast and lung cancer cell proliferation in vitro. SEMA3B inhibits VEGF₁₆₅ binding to receptors on these tumor cells and the effect can be mediated by NP receptors alone in the absence of VEGF receptor expression. These results suggest that tumor cell produced VEGF-A is a tumor cell survival or growth factor and that SEMA3B acts through a VEGF regulated system to mediate its tumor suppressor effects. These results are significant because it is well known that tumors require angiogenesis to be able to grow and survive.

Changes in future work

Screening studies in breast cancer for SEMA3B effect and methylation are important, but studies in SEMA3B signaling pathways in tumor and normal cells are crucial. Our observations have proved to us that SEMA3B is a candidate as a new therapeutic agent for breast, lung and other cancers. However, we need to understand with clarity how SEMA3B action takes place in the cells and why the end result is apoptosis. At this point one of the leading pathways that we will like to look at is AKT/PKB pathways.

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Semaphorin 3B (SEMA3B) induces apoptosis in lung and breast cancer, whereas VEGF₁₆₅ antagonizes this effect

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Semaphorin 3B (SEMA3B) is a secreted member of the semaphorin family, important in axonal guidance. We and others have shown that SEMA3B can act as a tumor suppressor by inducing apoptosis either by reexpression in tumor cells or applied as a soluble ligand. The common method of inactivation of SEMA3B is by allele loss and tumor-acquired promoter methylation. We studied the mechanism of SEMA3B-induced tumor cell apoptosis and found that vascular endothelial growth factor (VEGF)₁₆₅ significantly decreased the proapoptotic and antimitotic effect of transfected or secreted SEMA3B on lung and breast cancer cells. VEGF₁₆₅ binds to neuropilin, receptors for SEMA3B, and we found that SEMA3B competed for binding of ¹²⁵I-VEGF₁₆₅ to lung and breast cancer cells. We also found that small interfering RNA knockdown of tumor-produced VEGF-A or the use of an anti-VEGF neutralizing antibody (Ab) significantly inhibited tumor cell growth *in vitro*. By contrast, VEGF₁₂₁, a VEGF variant that lacks binding to neuropilin (NP)-1 or NP-2 receptors, was not expressed in tumor cells and had no effect on SEMA3B growth-suppressing activities. In conclusion, we hypothesize that VEGF₁₆₅, produced by tumor cells, acts as an autocrine survival factor and that SEMA3B mediates its tumor-suppressing effects, at least in part, by blocking this VEGF autocrine activity.

Semaphorin 3B (SEMA3B) is located at 3p21.3, a site of very frequent allele loss and/or promoter methylation in the early pathogenesis of lung and breast cancer (1–3). SEMA3B encodes a protein with tumor suppressor activity for lung cancer (4). Treatment with exogenously added SEMA3B or introduction of a plasmid encoding SEMA3B into H1299 non-small cell lung cancer (NSCLC) cells led to induction of apoptosis and a dramatic decrease in colony formation (2, 4). By contrast, tumor-acquired SEMA3B missense mutations have lost this activity. Independent studies in ovarian cancer by Tse *et al.* (5) demonstrated that SEMA3B also inhibited ovarian tumor formation in a xenograft model. Additionally, expression of SEMA3B in a p53-negative glioblastoma cell line was increased after reexpression of p53, suggesting that SEMA3B might also act as a mediator of p53 tumor-suppressor activity (6). However, because H1299 cells are p53 null, SEMA3B can induce marked tumor suppression even in the absence of p53.

SEMA3B is a secreted protein that belongs to the class 3 semaphorins (7, 8). Although the major role of SEMA3 proteins is to guide axons, they are also involved in diverse processes such as immune modulation (9, 10), organogenesis (11), neuronal apoptosis (12), and drug resistance (2, 13). SEMA3 members form complexes with two types of cell surface receptors: neuropilins (NP-1 and NP-2) and plexins (14–16). Neuropilins provide binding sites for SEMA3 whereas plexins are necessary for signal transduction by SEMA3B (17–19). In addition to semaphorins, NP-1 and NP-2 also bind several members of the vascular endothelial growth factor (VEGF) family, including VEGF-A (20, 21), VEGF-B, and placental growth factor (PIGF) (22, 23), all of which are angiogenic factors. VEGF-A is an endothelial cell (EC) agonist and is essential for vasculogenesis, angiogenesis, and wound healing and plays a role in tumor

angiogenesis. VEGFR-1 (Flt-1) and VEGFR-2 (KDR) are the classic receptors for VEGF-A. VEGF-A isoforms (e.g., VEGF₁₆₅ and VEGF₁₂₁) are created by alternative mRNA splicing and possess identical affinity for all VEGF receptors (24). By contrast, NPs bind VEGF₁₆₅ but not VEGF₁₂₁, the shortest isoform that lacks a heparin-binding domain (20). *In vitro* studies show that VEGF₁₆₅ interaction with NPs enhances VEGF-A effects, transduced through VEGFR-2, such as chemotaxis, EC survival, and angiogenesis (21, 25). *In vivo* studies show that NP-1 overexpression or silencing leads to abnormalities in blood vessel formation and the cardiovascular system during embryogenesis, indicating the importance of NP-1 interaction with the members of the VEGF family during development (26).

SEMA3A has been the most studied protein of the SEMA3 subclass due to its demonstrated role in axon guidance. It has been suggested that VEGF₁₆₅ is able to antagonize the proapoptotic and inhibitory effects of SEMA3A in the nervous system because both proteins share the same binding domain on cell-surface receptors (27). Porcine and rat aortic endothelial cell expressing NP-1 and VEGFR-2 respond to exogenously added SEMA3A by decreasing cell migration, as well as microvessel and lamellipodia formation (28). These effects were abolished by VEGF₁₆₅ (28). A similar pattern is observed in medulloblastoma cells expressing NP-1 and VEGFR-1; SEMA3A induces apoptosis in these cells whereas VEGF₁₆₅ antagonizes this effect by promoting cell proliferation and survival (29). VEGFs are indirect promoters of tumor growth *in vivo* by inducing angiogenesis that is crucial for supporting expansion of tumor mass and metastases. In addition, members of the VEGF family support tumor growth directly by acting as autocrine survival factors for those malignant cells that express VEGF or NP receptors (30–32). VEGF and SEMA3A are antagonistic autocrine NP-1 ligands that regulate breast carcinoma cell migration (33). The existence of common receptor(s) for both VEGF and semaphorin(s) implies that these factors might compete for the same binding sites on the cell surface.

Tumor cells produce VEGF, which could act as an autocrine survival factor as well as stimulate tumor angiogenesis. Because SEMA3B exerts an anti-tumor, antiproliferative and proapoptotic effect on lung and ovarian cancer cells *in vitro*, we hypothesized that SEMA3B effects on tumor cell growth and viability might be due to its ability to block autocrine VEGF survival pathway by competing for the same receptor(s). The present study was undertaken to characterize the interacting effects of

Abbreviations: SEMA3B, semaphorin 3B; NSCLC, non-small cell lung cancer; VEGF, vascular endothelial growth factor; VEGFR, VEGF receptor; NP, neuropilin; CM, conditioned medium; HUVEC, human umbilical vein endothelial cells; siRNA, small interfering RNA.

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SEMA3B activity and VEGF₁₆₅ in NSCLC and breast cancer. Our findings indicate that SEMA3B causes apoptosis in these two common human cancers and that this effect can be overridden by VEGF₁₆₅. These data imply an interacting role of these two proteins during tumorigenesis, providing a mechanism for the tumor-suppressing activity of SEMA3B.

Materials and Methods

Materials. We obtained human VEGF (VEGF₁₆₅) from Pepro-Tech (Rocky Hill, NJ), tissue culture and transfection materials from Invitrogen, ¹²⁵I-labeled VEGF₁₆₅ from PerkinElmer Life Science, chemiluminescence film (Hyperfilm ECL) from Amersham Pharmacia, and other chemicals from Sigma, Invitrogen, and Bio-Rad.

Antibodies (Ab). Rabbit polyclonal and mouse monoclonal Ab were generated by standard methods using a mixture of three peptides (rabbit) derived from the sequence of human SEMA3B [CGH-RAEEPVLRL; CGRIEDGKSGSPYDPRHRAA (peptide for mouse monoclonal); CALQSLPLESRKGRNRRTTHAPEP] as described in Tomizawa *et al.* (4). The SEMA3B rabbit polyclonal serum containing the Ab was used as a neutralizing agent, and the SEMA3B mouse monoclonal Ab was used for Western blots. Preimmunized rabbit serum was used as a control. SEMA3B rabbit polyclonal Ab specificity was determined by using transfected cells with SEMA3B, and neutralization ability was studied by cells treated with or without SEMA3B-CM. Mouse anti-human VEGF-A monoclonal Ab were purchased from Oncogene (Ab-5 for neutralization and Ab-3 for Western blot). Anti-VEGF 2C3 Ab is a mouse monoclonal Ab that inhibits the binding of human VEGF-A to receptors (VEGFR-2 and neuropilins). Pro-caspase 3 and cleaved caspase-3 Ab were purchased from Cell Signaling Technology (Beverly, MA).

Cell Lines. Cell lines included lung cancers NCI-H1299, NCI-H157, and HCC44 and breast cancer cell lines H1806, HCC1569, HCC1437, and HCC2185 from the Hamon Center Repository; Cos7 monkey kidney cells and breast cancer cells MDA-MB-231 from the American Type Culture Collection (ATCC); and human umbilical vascular endothelial cells (HUVEC) from Clonetics (San Diego). Lung cancer cells and Cos-7 were grown in RPMI medium 1640 supplemented with 5% FBS (R5 medium). HUVEC were grown in Endothelial Cell Basal Media from Cambrex (East Rutherford, NJ), and MDA-MB-231 cells were grown in MEM supplemented with L-glutamine and 10% FBS.

Expression Plasmids. Genes encoding human wild-type (WT) *SEMA3B*, mutant *SEMA3B* containing single missense mutations D397H (SEMAMUT1) and T415I (SEMAMUT2), and WT p53, VEGF₁₂₁, and VEGF₁₆₅ were inserted into pcDNA3 expression vector (Promega).

RT-PCR. Total RNA was extracted by using RNeasy Mini kit (Qiagen, Valencia, CA). RT-PCR was performed by using the SuperScript One Step RT-PCR Systems (Invitrogen), and amplification products were resolved on 1% agarose gels. A schedule for typical RT-PCR consisted of 1 h of reverse transcription at 42°C, 1 min of denaturation at 95°C, 1 min of annealing, and 1 min of extension at 72°C. All samples analyzed by RT-PCR were also tested for GAPDH expression to confirm the integrity of the RNA.

Small Interfering RNA (siRNA). The siRNA (sense and antisense strands) was obtained from the Center for Biomedical Interventions (University of Texas Southwestern Medical Center). The sense strands sequences were the following: VEGF, 5'-AUGUGAAUGCAGACCAAGAATT and control, 5'-

GAUAGACAAAUGACGAAUGCGUATT. *In vitro* transfection was performed by using the Oligofectamine reagent from Invitrogen. Thirty percent confluent cells in six-well plates were treated with 100 nM siRNA, the cells were washed after 6 h, and the experiment ended after 72 h.

Western Blot Analysis. Cell extracts were made in Nonidet P-40 extraction buffer (40 mM Hepes-NaOH, pH 7.4/1% Nonidet P-40/0.5% sodium deoxycholate/0.1% SDS/150 mM NaCl/protein inhibitors), and whole cell extracts (50–75 µg of protein) were separated on 10% SDS/PAGE gels and transferred to Hybond-P membrane (Schleicher & Schuell). Membranes were blocked for 30 min with 5% dry milk in 0.1% Tween 20 in Tris-buffered saline, incubated at room temperature for 2 h with monoclonal anti-SEMA3B Ab, then 40 min with horseradish peroxidase-labeled anti-mouse IgG (Amersham Pharmacia), and developed by Super Signal Chemiluminescence substrate (Pierce).

Cos7 Conditioned Medium (CM) Preparation. Cos7 cells were transfected with the vector pcDNA3 (negative control), or plasmids encoding SEMA3B, SEMA3Bmut, VEGF₁₂₁, and VEGF₁₆₅. Medium was collected 48 h posttransfection. SEMA3B present in the medium was determined by Western blot and by neutralizing its effect with the anti-SEMA3B rabbit polyclonal Ab. Semiquantitative assay showed an average of 15–40 ng/ml SEMA3B in the CM after transfection. Levels of VEGF isoforms and SEMA3B in the medium were determined by Human VEGF Accucyte EIA kit (Oncogene, San Diego, CA) and Western Blot, respectively.

Binding of ¹²⁵I-VEGF₁₆₅ to MDA-MB-231, H157, and H1299 Cells. Cells were seeded in 12- or 24-well plates and allowed to grow to confluence (2–5 × 10⁵ cells per well). Cells were washed with cold PBS followed by washing with binding buffer (DMEM/0.1% gelatin/20 mM Hepes, pH 7.2/1 µg/ml heparin). To determine nonspecific binding, cells were preincubated with 100-fold molar excess of cold VEGF₁₆₅ or SEMA3B-CM for 30 min at 4°C. ¹²⁵I-VEGF₁₆₅ (80–100 pM) was added and incubated for 2 h at 4°C, cells were washed three times with cold PBS containing 0.1% BSA (BSA-PBS), the plates were shaken with lysis buffer (1% Triton X-100 in 0.1% BSA-PBS) for 30 min at room temperature, and 350 µl from each well was counted in a Cobra II Auto Gamma counter (Packard).

Transfections. Cells were transfected by using Lipofectamine (Invitrogen) according to the manufacturer's instructions and analyzed 48 h after transfection.

Cell Growth Assay Using the Cos7 CM. H1299 and MDA-MB-231 cells were seeded in six-well plates at a density of 10,000 cells per well in the presence of CM from Cos7 cells, transfected either with vector, SEMA3B, SEMA3Bmut, VEGF₁₂₁, or VEGF₁₆₅, diluted 1:2 with medium, and cells were counted 5 days later. Assays done in triplicate were repeated at least two times.

Cell-Cycle Analysis. Cells were harvested 72 h after transfection, fixed with 70% ethanol, treated with 5 mg/ml RNase A (Sigma), stained with 50 µg/ml propidium iodide, and analyzed by flow cytometry for DNA synthesis and cell-cycle status (FACSCalibur instrument and CELLQUEST software, Becton Dickinson).

Results

Expression of VEGFR-1, VEGFR-2, NP-1, NP-2, and VEGF Isoforms in NSCLC. Because SEMA3B was likely to compete with endogenously expressed VEGF-A for VEGF receptors R1 and R2 as well as NP-1 and NP-2, we determined, by using RT-PCR, which isoforms of VEGF-A and which receptors are expressed in the

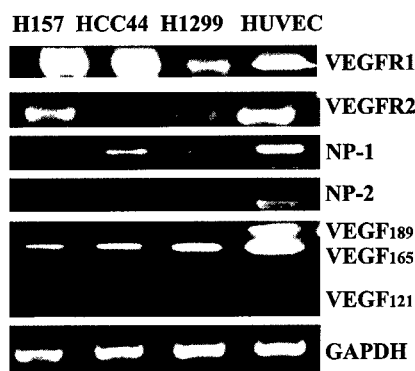


Fig. 1. Expression of VEGFR-1, VEGFR-2, NP-1, NP-2, and VEGF-A isoforms in lung cancer cells and HUVEC. RNA was collected from dividing cells, and the presence or absence of VEGF-A isoforms and receptors was determined by RT-PCR. Primers to GAPDH were used to confirm integrity of RNA. HUVEC were used as a positive control for gene expression.

human tumor cells selected for functional studies. HUVEC expressed all of the genes and was used as a positive control (Fig. 1) (34, 35). VEGFR-1 and NP-1 were detected in all three NSCLC lines whereas none of the lines expressed NP-2; VEGFR-2 was detected in NSCLC H157 and H1299 cells but not in NSCLC HCC44 cells; and all NSCLC lines expressed VEGF₁₆₅ but not VEGF₁₂₁ or VEGF₁₈₉ isoforms (Fig. 1). Determined by immunoassay, H1299 and Cos7 cells both endogenously produced $10\text{--}12 \pm 1$ ng/ml VEGF₁₆₅ into the culture media under control conditions. By contrast, VEGF₁₆₅ plasmid transfected H1299 cells secreted $\approx 200 \pm 15$ ng/ml VEGF₁₆₅ into culture medium (a 20-fold increase in production compared with endogenous levels).

VEGF-A Plays a Role in the Survival of H1299 Lung Cancer Cells. H1299 cells express detectable amounts of VEGF-A under normal culture conditions. We wished to determine the effect on H1299 cell proliferation after VEGF-A removal. We have used two approaches to this, siRNA knockdown of VEGF-A and an anti-VEGF-A neutralizing Ab (Ab-5). By using techniques that remove or neutralize the biological activity of VEGF-A, we observed a $>50\%$ decrease in cancer cell proliferation after 3 days of treatment or transfection (Fig. 2*A* and *B*). A decrease in endogenous VEGF protein levels was seen after transfection of VEGF siRNA but not after control siRNA treatment (Fig. 2*C*). These data suggest that tumor cell produced VEGF has an important role in the survival or growth of NSCLC H1299.

Presence of SEMA3B Protein in the CM from Cos7 Cells. CM derived from Cos7 cells transfected with the SEMA3B expression vector and purified on an anti-SEMA3B Ab column showed a band that reacted with anti-SEMA3B Ab (Fig. 3). We collected the Cos7 cell-derived CM containing SEMA3B (SEMA3B-CM) at 48 h posttransfection to use as a source of exogenously added SEMA3B protein in all assays described below. The medium from Cos7 cells transfected with empty vector (Control-CM) was used as a negative control. We used this affinity-purified protein to estimate that $15\text{--}40$ ng/ml SEMA3B was in the CM.

SEMA3B Inhibits ¹²⁵I-VEGF₁₆₅ Binding to Lung and Breast Cancer Cells. Cell-surface binding of ¹²⁵I-VEGF₁₆₅ was tested on the NSCLCs H1299 and H157 and breast cancer MDA-MB-231 cells (Fig. 4) with and without the addition of SEMA3B. The MDA-MB-231 line was chosen because these cells express NP-1 and NP-2 but not VEGFR-1 or VEGFR-2, thus enabling us to distinguish between signaling pathways induced by different VEGF receptors. Specific ¹²⁵I-VEGF₁₆₅ binding was observed in all cell lines

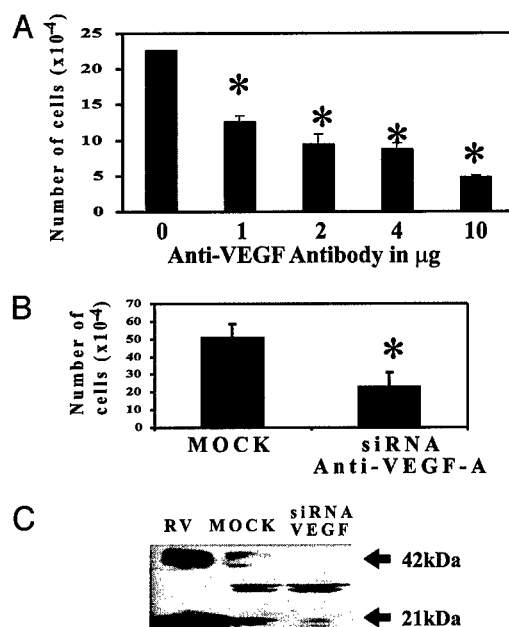


Fig. 2. VEGF-A plays a role in the survival of H1299 lung cancer cells. H1299 cells were seeded and treated with anti-VEGF Ab or transfected with siRNA for VEGF-A (see *Materials and Methods*). (A) Quantification of cell proliferation after H1299 cells were treated with anti-VEGF Ab for 4 days. The Ab inhibition of cell proliferation was dose-dependent. (B) Quantification of cell proliferation assay after H1299 cells were transfected with siRNA control (MOCK) and VEGF-A. (C) Western blot analysis for VEGF after 72 h of MOCK or VEGF siRNA transfection (180 μg of protein) and recombinant VEGF (RV) as a positive control. *, $P < 0.05$, *t* test.

after 2 h of incubation with 100 pM of labeled VEGF₁₆₅ (Fig. 4, lane 1). SEMA3B-CM decreased the specific binding of ¹²⁵I-VEGF₁₆₅ to all cell lines (Fig. 4, lanes 2–4) with maximal inhibition of 50% for MDA-MB-231 and 65–75% for H1299 and H157, respectively. By contrast, Cos7 control-CM had no significant effect on ¹²⁵I-VEGF₁₆₅ binding (Fig. 4, lane 5). These data suggest that VEGF₁₆₅ and SEMA3B share binding sites on these human tumor cells as was previously observed for SEMA3A and VEGF₁₆₅ on NP receptors (36).

VEGF₁₆₅ Antagonizes SEMA3B-Mediated Effects on Tumor Cell Proliferation. SEMA3B gene and protein expression was not detected in the majority of NSCLC, including H1299 and H157 (2, 4). SEMA3B-CM inhibited proliferation of H1299 cells by 50% compared with vector control whereas rabbit anti-SEMA3B Ab neutralized this effect (Table 1). Control IgG from preimmune rabbits had no effect on the inhibitory effect of the SEMA3B-CM or vector control (data not shown). These results demonstrate that SEMA3B protein present in the CM of SEMA3B-transfected Cos7 cells is responsible for the antiproliferative effect. Treatment with mutant SEMA3Bmut1 vector showed no effect in cell number when compared with CM. The antiproliferative effect of SEMA3B-CM was overridden by

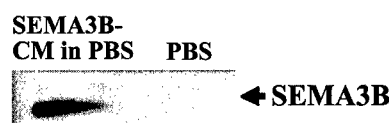


Fig. 3. Expression of SEMA3B protein after transfection and protein purification of Cos7 CM. Cos7 cells were transfected with SEMA3B expression plasmid. Shown is Western blot analysis for Cos7 CM after transfection with SEMA3B expression plasmid and anti-SEMA3B column protein purification.

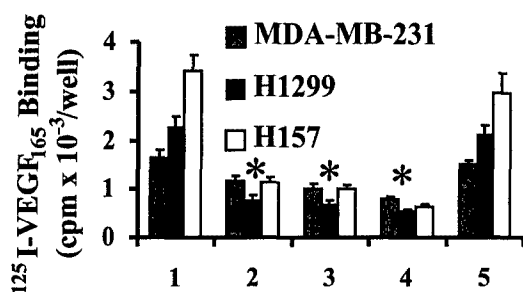


Fig. 4. Competition of SEMA3B and ¹²⁵I-VEGF₁₆₅ for binding to the cell surface receptors on MDA-MB-231, H1299, and H157 cells. MDA-MB-231 (gray), H1299 (black), and H157 (white) cells were incubated for 2 h with ¹²⁵I-VEGF₁₆₅ and SEMA3B-CM in the binding buffer, and the bound ¹²⁵I-VEGF₁₆₅ was detected by using a γ counter. ¹²⁵I-VEGF₁₆₅ binding with SEMA3B-CM added at 5%, 15%, and 30% of total volume per well (200 μ l) (lanes 2–4), which CM from the Cos7 cells transfected with empty vector served as a control (lane 5). Nonspecific binding was determined in the presence of 100-fold molar excess of unlabeled VEGF and was subtracted from the experimental values. We observed a nonspecific binding ranging from 12% to 40% when compared with specific binding from ¹²⁵I-VEGF₁₆₅ alone.

cotreatment with VEGF₁₆₅-CM. By contrast, cotreatment with VEGF₁₂₁, which does not bind to NP-1 and NP-2, had no significant effect on cell proliferation as compared with SEMA3B-CM alone. Transfection with VEGF₁₂₁ or VEGF₁₆₅ alone did not affect cell proliferation in the absence of SEMA3B-CM. The specificity of the VEGF₁₆₅ reversal of SEMA3B growth inhibition was confirmed by treating with monoclonal anti-VEGF Ab 2C3 (37), which gave similar cell numbers as treatment with SEMA3B alone. In contrast to the anti-VEGF neutralizing Ab Ab-5, the anti-VEGF Ab 2C3 does not inhibit cell proliferation. The lack of effect of VEGF₁₂₁ indicates that only a NP-binding VEGF isoform (VEGF₁₆₅) is able to counteract the effects of SEMA3B.

Similarly, transfection with SEMA3B led to an 65–80% decrease in colony number for H1299 and H157 cells compared

with untreated cells or cells transfected with empty vector (H1299 vector control 200 ± 18 , SEMA3B transfected 42 ± 5.1 , H157 vector control 230 ± 2.3 , and SEMA3B transfected 78 ± 7.2 colonies, respectively). When cells were cotransfected with both VEGF₁₆₅ and SEMA3B plasmids, the number of colonies was not significantly different from that in the control cells whereas cotransfection of SEMA3B and VEGF₁₂₁ showed reduced colony number, indicating that VEGF₁₂₁ isoform does not modulate the inhibitory effect of SEMA3B (H1299 SEMA3B-VEGF₁₂₁ 44 ± 7 colonies and for H157 85 ± 11 colonies). Taken together, these data suggest that SEMA3B is a potent growth inhibitory factor for human NSCLC cells and that VEGF₁₆₅, but not the VEGF₁₂₁ isoform, overcomes the SEMA3B growth inhibitory effect.

The Effect of SEMA3B and VEGF₁₆₅ on MDA-MB-231 Breast Cancer Cells Lacking VEGFR-1 and VEGFR-2. Growth inhibition of 50–60% by SEMA3B-CM was also observed in five breast cancer lines (see *Materials and Methods* for list of cell lines). In contrast to NSCLC cells described above that express NP-1 as well as one or both classic VEGF receptors, MDA-MB-231 breast cancer cells express only NP-1 and NP-2 proteins (20, 21). We confirmed by RT-PCR that MDA-MB-231 does not express VEGFR-1 or VEGFR-2. We used this line to demonstrate that SEMA3B induced growth inhibition and that VEGF₁₆₅ reversal of this effect can be mediated through neuropilin receptors alone and does not have to involve VEGFR-1 or VEGFR-2 (Table 1). Thus, treatment with SEMA3B-CM caused a 50% reduction in MDA-MB-231 cell number (while SEMA3Bmut-CM was inactive) and the rabbit anti-SEMA3B Ab (10 μ g/ml) blocked the SEMA3B-CM growth inhibition. VEGF₁₆₅ (but not VEGF₁₂₁) reversed the SEMA3B growth inhibition, which in turn was neutralized by the anti-VEGF Ab 2C3. When the anti-VEGF Ab 2C3 Ab was added to MDA-MB-231 cells in the absence of SEMA3B but in the presence of VEGF₁₆₅, it had no effect on cell growth, and VEGF₁₆₅ or VEGF₁₂₁ treatment alone did not alter breast cancer cell growth (Table 1). Taken together, these data show that SEMA3B-CM has an antiproliferative effect on

Table 1. Effect of SEMA3B-CM and VEGF isoforms 165 and 121 on proliferation in H1299 lung and MDA-MB-231 breast cancer cells

Treatment*	H1299 Cell no. ($\times 10^{-4}$) (P value) [†]	MDA-MB-231 Cell no. ($\times 10^{-4}$) (P value) [†]
Control-CM [‡]	11 \pm 1.3 (–)	16 \pm 1.0 (–)
SEMA3B-CM [‡]	6 \pm 0.6 (0.002) [§]	5 \pm 2.0 (0.002) [§]
SEMA3Bmut1-CM [‡]	11 \pm 1.0 (0.0005)	15 \pm 0.5 (0.0005)
SEMA3B-CM plus anti-SEMA3B**	11 \pm 1.5 (0.004)	13 \pm 1.0 (0.004)
VEGF ₁₂₁	11 \pm 0.8 (NS) ^{††}	16 \pm 1.0 (NS) ^{††}
VEGF ₁₆₅	11 \pm 0.8 (NS) ^{††}	16 \pm 2.0 (NS) ^{††}
VEGF ₁₂₁ plus SEMA3B-CM	6 \pm 1.2 (0.004) ^{††}	8 \pm 0.3 (0.003) ^{††}
VEGF ₁₆₅ plus SEMA3B-CM	12 \pm 1.5 (0.0002)	15 \pm 0.6 (0.003)
VEGF ₁₆₅ plus anti-VEGF-A Ab	12 \pm 0.9 (NS) ^{§§}	14 \pm 0.8 (NS) ^{§§}
VEGF ₁₆₅ plus SEMA3B-CM plus anti-VEGF Ab**	7 \pm 0.6 (0.001) [¶]	4 \pm 0.9 (0.001) [¶]

*CM from cells transfected with various plasmids as specified above was added to MDA-MB-231 and H1299 cells.

[†]P value was calculated by using a two-tailed Student's *t* test.

[‡]Cos7 cells were transfected with vector control or with SEMA3B expression vector, and CM was collected 48 h thereafter.

[§]Significant decrease as compared with Control-CM.

^{||}SEMA3B-mut-CM (D397H) is inactive as described in *Materials and Methods* and *Results*.

^{||}Significant increase as compared with SEMA3B-CM.

^{**}Anti-SEMA3B or anti-VEGF (30 μ g/ml) antibody 2C3 was used as neutralizing agent for SEMA3B-CM and VEGF₁₆₅, respectively.

^{††}Nonsignificant as compared with control.

^{††}Significant decrease when compared with cells treated with VEGF₁₂₁.

^{§§}Nonsignificant as compared with VEGF₁₆₅.

^{||¶}Significant decrease as compared with VEGF₁₆₅ plus SEMA3B-CM.

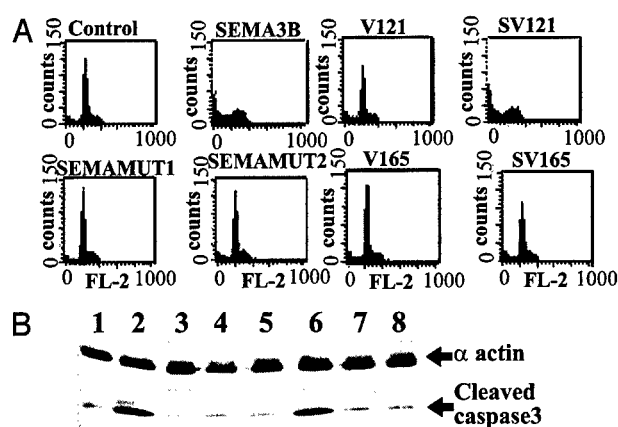


Fig. 5. SEMA3B induces caspase-3-dependent apoptosis and VEGF₁₆₅ antagonizes this effect. (A) Flow cytometry analysis of apoptosis induction in H1299 cells 72 h posttransfection. NSCLC H1299 cells were transfected with pcDNA3 (vector control) SEMA3B, VEGF₁₂₁ (V121), SEMA3B plus V121 (SV121), SEMAMUT1, SEMAMUT2, VEGF₁₆₅ (V165), and SEMA3B plus V165 (SV165), and FACS assay was performed to determine induction of apoptosis. The number of tumor cells undergoing apoptosis are indicated by sub G₀ peak. (B) Caspase-3 cleavage increase detected by Western blotting as a hallmark of apoptotic activation. Vectors used in transfection of H1299 cells are as follows: lane 1 (vector control), lane 2 (SEMA3B), lane 3 (SEMAMUT1), lane 4 (SEMAMUT2), lane 5 (V121), lane 6 (SEMA3B plus V121), lane 7 (V165), and lane 8 (SEMA3B + V165).

MDA-MB-231 breast cancer cells expressing NP-1 and NP-2 but not VEGFR-1 and VEGFR-2, and that VEGF₁₆₅ but not VEGF₁₂₁ is able to antagonize this effect.

SEMA3B Induces Apoptosis of H1299 Lung Carcinoma Cells and VEGF₁₆₅ Abolishes This Effect. We examined whether VEGF₁₆₅ could decrease apoptosis induced in lung cancer cells by SEMA3B by using fluorescence-activated cell sorter (FACS) analysis and caspase 3 activation assays (4) (Fig. 5). H1299 cells were transfected with vector control and SEMA3B for 72 h in the presence or absence of cotransfected VEGF₁₂₁ or VEGF₁₆₅. More than 94% of the vector control cells were alive by the end of the 72-h period, with only 6% of cells displaying signs of apoptotic death (Fig. 5A). As expected, SEMA3B treatment increased the number of tumor cells in apoptosis (sub G₀ peak, Fig. 5A) by 40%. VEGF₁₂₁ and VEGF₁₆₅ given alone did not alter apoptosis or the cell cycle. Cotransfection of VEGF₁₆₅ with SEMA3B reversed the effect of SEMA3B on apoptosis induction whereas cotransfection with VEGF₁₂₁ did not (Fig. 5A). Increase in the caspase-3 activity that arises after the cleavage of pro-caspase-3 is a hallmark of induced apoptosis and is considered to be a point of no return. Transfection of H1299 cells with SEMA3B increased caspase-3 activity relative to basal levels in the control cells whereas SEMA3B mutants had no effect (Fig. 5B). However, cotransfection of SEMA3B with VEGF₁₆₅ antagonized the SEMA3B effect on caspase-3 cleavage (Fig. 5B). These data demonstrate that SEMA3B induced caspase-3-dependent apoptotic pathway in H1299 cells and that this effect was reversed by VEGF₁₆₅.

Discussion

SEMA3B, encoded at 3p21.3, is a candidate tumor suppressor gene (4, 5). Evidence in support of this finding includes the following: frequent allele loss at the *SEMA3B* 3p21.3 locus; frequent loss of *SEMA3B* expression in lung cancer secondary to tumor-acquired promoter methylation; occasional somatically acquired tumor *SEMA3B* mutations; and inhibition of tumor cell growth *in vitro* and *in vivo* coupled with dramatic ability to induce tumor cell apoptosis after transfection or exposure to SEMA3B protein (3–5). In the

present work, we have shown that reexpression of WT but not mutant SEMA3B induces apoptosis in lung and breast cancer cells and that this effect is reversed by coexpression of VEGF₁₆₅ but not VEGF₁₂₁. We also have shown that siRNA-mediated VEGF knockdown or neutralizing anti-VEGF Ab inhibit lung cancer cell proliferation *in vitro*. SEMA3B inhibits VEGF₁₆₅ binding to receptors on these tumor cells, and the effect can be mediated by NP receptors alone in the absence of VEGF receptor expression. These results suggest that tumor cell-produced VEGF-A is a tumor cell survival or growth factor and that SEMA3B acts through a VEGF-regulated system to mediate its tumor suppressor effects. These findings accord with prior observations by Bachelder *et al.* that NP-1 supports a VEGF signaling pathway that is critical for breast carcinoma cell survival (32) and that VEGF and SEMA3A are antagonistic NP-1 ligands that regulate breast carcinoma cell migration (33). Also, SEMA3A induces apoptosis in neurons (38, 39), competes with VEGF-A binding on porcine aortic endothelial, medulloblastoma, and human embryonic kidney 293 cells, and affects cell survival and migration (27–29). Recent research also shows SEMA3F (whose gene is located \approx 70 kb telomeric of *SEMA3B* in the same 3p21.3 region) and VEGF as having opposing effects on cell attachment and motility in breast cancer lines MCF-7 and C100 (40). In these studies, SEMA3F inhibited lamellipodia formation, membrane ruffling, and cell-cell contacts through interaction with NP-1 (40).

Based on these observations and prior findings, it seems that SEMA3B functions as a suppressor of tumor growth by inducing apoptosis potentially in premalignant as well as malignant cells. Additional support for our hypothesis came when a p53-negative glioblastoma cell line after reintroduction of p53 showed increased expression of SEMA3B. Furthermore, UV radiation and doxorubicin treatment also induced SEMA3B in MCF-7 breast cancer cells (6). These findings suggested that SEMA3B could be mediating a p53 DNA damage response. However, SEMA3B induces apoptosis in the NSCLC H1299 cell line, which is null for p53, demonstrating that reexpression of SEMA3B alone is sufficient for induction of apoptosis. Because SEMA3B and VEGF₁₆₅ share similar binding sites on NP-1 and NP-2 proteins, we also propose that VEGF, which is frequently expressed in human cancers including lung and breast cancer (41–45), would compete with SEMA3B for binding on the cell surface, and would antagonize the negative regulatory effects of SEMA3B on cell growth.

Based on the present findings and published observations, we propose the following working model. During the course of carcinogenesis, DNA damage and mutations occur, activating the p53 pathway, among other things, leading to increased expression of SEMA3B. SEMA3B induction would in turn lead to induction of apoptosis, removing damaged cells. However, premalignant cells that had undergone 3p21.3 allele loss, mutation, and/or SEMA3B promoter methylation become haploinsufficient for SEMA3B, or lose this control mechanism entirely. Likewise, expression of VEGF₁₆₅ by premalignant cells or by neighboring cells would bypass this effect. Thus, either or both loss of SEMA3B expression and/or VEGF₁₆₅ overexpression would lead to an outgrowth of cells with genetic abnormalities. However, in many lung and breast cancers, it seems the combination of loss of SEMA3B expression, VEGF overproduction, and p53 mutation occur together. This result would indicate that, even though these mechanisms are interrelated, they also have other separate growth control properties that must be eliminated for a clinically evident malignancy to develop. Finally, the ability of exogenously added SEMA3B to induce apoptosis in full-fledged tumor cells with numerous genetic and epigenetic changes and expressing endogenous VEGF₁₆₅ suggests the potential use of SEMA3B as a systemic therapeutic agent.

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